JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 6141–6148 6141
DOI:10.1021/jf100633f

Enzymatic Xylose Release from Pretreated Corn Bran Arabinoxylan: Differential Effects of Deacetylation and Deferuloylation on Insoluble and Soluble Substrate Fractions

Jane Agger,[†] Anders Viksø-Nielsen,[‡] and Anne S. Meyer*,[†]

[†]Bioprocess Engineering, Department of Chemical and Biochemical Engineering, Technical University of Denmark, Søltofts Plads 229, DK-2800 Kgs. Lyngby, Denmark, and [‡]Novozymes A/S, Bagsværd, Denmark

In the present work enzymatic hydrolysis of arabinoxylan from pretreated corn bran (190 °C, 10 min) was evaluated by measuring the release of xylose and arabinose after treatment with a designed minimal mixture of monocomponent enzymes consisting of α-L-arabinofuranosidases, an endoxylanase, and a β-xylosidase. The pretreatment divided the corn bran material ∼50:50 into soluble and insoluble fractions having A:X ratios of 0.66 and 0.40, respectively. Addition of acetyl xylan esterase to the monocomponent enzyme mixture almost doubled the xylose release from the insoluble substrate fraction and gave release of 1 mol of xylose/mol of acetic acid released, whereas addition of feruloyl esterase promoted release of only ∼0.4 mol of xylose/mol of ferulic acid released. For the soluble substrate fraction up to 36% of the xylose could be released by the enzymatic treatment. Acetyl xylan esterase addition on top of the minimal monocomponent enzyme mixture resulted in liberation of up to 0.5 mol of xylose/mol of acetic acid released, whereas feruloyl esterase addition released 1 mol of xylose/mol of ferulic acid released from the soluble substrate. The results imply that on the insoluble material the acetyl xylan esterase was more important for the enzymatic degradation than feruloyl esterase, whereas on soluble arabinoxylan the feruloyl esterase seemed to be more important for the release of xylose.

KEYWORDS: Enzymatic hydrolysis; acetyl xylan esterase; feruloyl esterase; arabinoxylan; corn bran; xylose

INTRODUCTION

In 2007 the Food and Agricultural Organization (FAO) of the United Nations reported that the total production of corn in the United States was 330×10^6 tons (*1*). Corn bran is an agro-industrial byproduct resulting from the wet milling step in corn starch processing and consists almost exclusively of the pericarp tissue, testa, and pedicel tip of the corn kernel (*2*). Considering that a large starch producer in the United States processes about 50000 tons of corn per day (*2*), with a cautious estimate of 5% (by weight) of this corn becoming corn bran, the yield of corn bran would be approximately 9×10^5 tons per year from this producer alone. As a clean and readily available agricultural residue, corn bran may have the potential of becoming a source for new C5 biofuel products or for the manufacture of food ingredients. These novel uses require partial or complete degradation of the biomass to its constituent monomers. The investigation of the chemical composition and the enzymatic degradability of corn bran is therefore an important objective of several current studies.

Corn bran originates from a gramineaceous plant having a primary cell wall that is mainly composed of heteroxylans (approximately 50% by dry weight), notably arabinoxylan (*3*). Much effort has been put into describing the complexity of arabinoxylan from corn bran and into understanding the interactions between arabinoxylan and other cell wall components (*3*−*6*). A simplified sketch of the corn bran arabinoxylan structure is shown in **Figure 1**: The arabinoxylan backbone in corn bran is composed of a xylan backbone of β-(1→4)-linked D-xylopyranosyl residues. Linkage analysis has suggested that up to 85% of the xylopyranosyl moieties are substituted with various components (*4*). The main substitutions are α-L-arabinofuranosyl residues linked to the O-2 or O-3 position on monosubstituted xylopyranosyls (40%) or to both O-2 and O-3 on doubly substituted xylopyranosyl units (20%). Arabinofuranosyl substitutions have also been suggested to occur as short side chains. As much as 40% of the total arabinofuranosyl substitutions have been reported as nonterminal (*4*). It has also been suggested that xylan is further substituted with xylopyranosyls by a (1→3)-linkage and that the arabinofuranosyls can be further decorated with xylopyranosyls or even L-galactopyranosyls (*5*, *6*). Xylan may furthermore be directly substituted with D-galactopyranosyl and D-glucuronyl residues, which may each account for approximately 3−5% of the biomass dry weight (*3*, *6*).

Besides glycosidic linkages, arabinoxylan is also substituted by esterifications. Acetic acid is found to constitute 3−5% of the corn bran dry matter; this acetic acid is esterified directly to the

*Author to whom correspondence should be addressed (telephone +45 4525 2909; fax +45 4588 2258; e-mail am@kt.dtu.dk).

Published on Web 04/22/2010

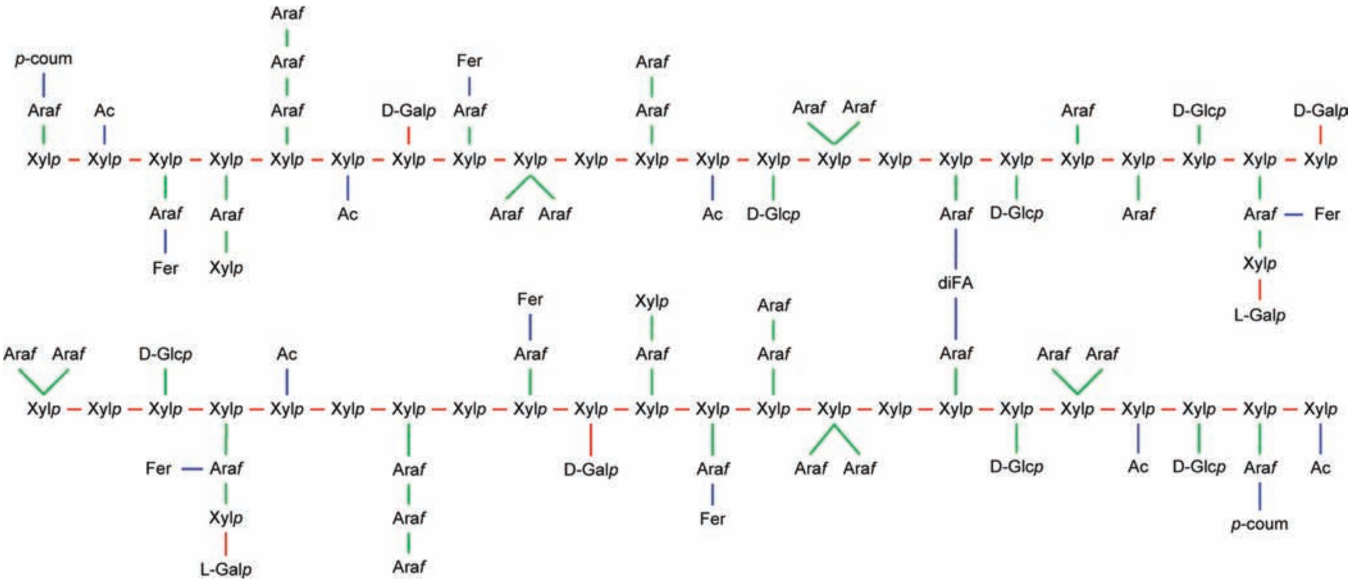

Figure 1. Simplified sketch of arabinoxylan structure from corn bran. Xyl*p*, xylopyranosyl residue; Ara*f*, arabinofuranosyl residue; D-Gal*p*, D-galactopyranosyl residue; L-Gal*p*, L-galactopyranosyl residue; D-Glc, D-glucuronsyl residue; Fer, feruloyl residue; Ac, acetyl residue; diFA, dehydrodimer feruloyl residue (any dimerization structure); *p*-coum, *p*-coumaroyl residue; red linkage, β-glycosidic linkage; green linkage, α-glycosidic linkage; blue linkage, ester linkage.

xylan backbone in position O-2 or O-3, whereas hydroxycinnamic acids (3−6% of the biomass dry weight) are esterified to arabinofuranosyls in position O-5 (*5*, *7*). The hydroxycinnamates are mainly *p*-coumaric acid, ferulic acid, and dehydrodimers of ferulic acid (*5*, *8*−*10*). Suggestions have even been made that feruloyl groups can be positioned on arabinofuranosyl in the previously mentioned short heterogeneous side chains rather than terminally on the arabinofuranosyl moiety (*5*). These side chains have commonly been referred to as FAX and FAXG. Also, trimers and tetramers of ferulic acid have been isolated from corn bran (*11*). It is known that, in particular, diferulates cross-link arabinoxylan molecules, thereby creating covalent intermolecular relations (*10*, *12*, *13*). These interactions have been shown to impede the enzymatic degradation of corn bran (*14*, *15*). Finally, corn bran also contains cellulose (~20%), lignin (~10−14%), and structural proteins (~5%). The lignin and structural proteins have been suggested to also participate in intermolecular interactions with arabinoxylan through diferulate cross-linking, giving rise to a highly complex network of heterogeneous molecules (*6*, *15*, *16*, *17*).

With respect to enzymatic digestibility, corn bran has been acknowledged as a recalcitrant substrate with little release of monosaccharides despite the application of several mixed enzyme activities at high dosage (*18*, *19*). To achieve reasonable enzymatic hydrolysis, hydrothermal pretreatment has been applied with success (*19*, *20*). The recalcitrance to degradation has been ascribed to be a consequence of the highly branched structure of the arabinoxylan, and indeed feruloylation has been held responsible (*21*, *22*). Remarkably, acetyl substitution also occurs in corn bran to the same extent as feruloylation, but the role of acetylation has been given very little attention in relation to enzymatic degradation of corn bran, even though early studies with other substrates such as wheat and aspen xylans have shown that enzymatic digestibility of both cellulose and xylan increased remarkably with increasing chemical deacetylation (*23*). A direct correlation between the enzymatic release of xylose and concomitant deacetylation of xylan has been reported for purified beech- and birchwood xylans (*24*, *25*). Hence, we hypothesized that deacetylation could be important for the overall enzymatic digestibility of xylan. The purpose of the present work was to assess and compare the overall release of xylose from corn bran arabinoxylan using relevant monocomponent enzyme activities and thereby to obtain indications as to the significance of deacetylation for enzymatic xylose release from corn bran arabinoxylan.

Table 1. Enzymes Used for Hydrolysis of Pretreated Corn Bran[a]

enzyme	microorganism	family/type	EC no.	ref
endoxylanase	*Humicola insolens*	GH10	EC 3.2.1.8	*34*
β-xylosidase	*Trichoderma reesei*	GH3	EC 3.2.1.37	*34*
α-L-arabinofuranosidase	*Meripilus giganteus*	GH51	EC 3.2.1.55	*34*
α-L-arabinofuranosidase	*Humicola insolens*	GH43	EC 3.2.1.55	*34*
acetyl xylan esterase (AXE)	*Flavolaschia* sp.	CE1	EC 3.1.1.72	
feruloyl esterase (FAE)	*Aspergillus niger*	type A, CE1	EC 3.1.1.73	*35*, *36*
Cellic CTec	*Trichoderma reesei*			

[a] All enzymes were provided by Novozymes A/S, Bagsværd, Denmark.

MATERIALS AND METHODS

Substrate. Raw corn bran was provided by Archer Daniel Midlands Co., Decatur, IL. The material was milled, enzymatically destarched, freeze-dried, and milled again before use. Initial wet milling was performed at 2% dry matter (DM), and destarching was carried out using a thermostable α-amylase (Termamyl SC, dosed at 7560 KNU-S/kg of corn bran) incubated at pH 6 and 95 °C for 0.5 h, followed by incubation with amyloglucosidase (Spirizyme Plus FG, dosed at 69000 AGU/kg of corn bran) at pH 5 and 60 °C for 1 h. After the enzymatic treatment, the material was washed in water and decanted to remove liberated glucose. The washing was performed three times, resulting in a free glucose concentration in the last volume of washing water below the detection limit of the HPAEC. Freeze-drying was done over 5 days, and the biomass was finally milled again to reduce the particle size to <1 mm. The destarched material was stored at −18 °C.

Enzymes. All enzymes were provided by Novozymes A/S, Bagsværd, Denmark. Cellic CTec is a commercially available cellulase preparation based on the *Trichoderma reesei* complex, whereas the others were monocomponent preparations (**Table 1**). Apart from the cellulolytic enzyme base from *T. reesei* containing at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, and a β-xylosidase (*26*, *27*), the preparation Cellic CTec also contains a particular proprietary hydrolysis-boosting protein.

Table 2. Monomeric Biomass Composition of Destarched Corn Bran (DCB) and Insoluble and Soluble Fractions of Pretreated Corn Bran[a]

		arabinoxylan								
		arabinose	xylose	cellulose	lignin	protein	ferulic acid	diferulic acid	acetic acid	weight distribution
DCB (g/kg of DM)		267	374	210	120	89.1	27.3	17.0	38.5	
insoluble (%)		17.5	29.5	73.4	76.4	57.4	21.4	24.7	23.8	45.6
soluble (%)	free	16.0	3.4	nd	nd	<dl	<dl	<dl	<dl	40.5
	bound	31.2	33.9	5.6	nd	14.4	37.4	1.9	55.9	
estimated loss[b] (%)		35.3	33.2	21	23.6	28.2	41.2	73.4	20.3	13.9

[a] DCB components are given as g/kg of DM. Insoluble, soluble, and estimated loss are given as a percent of individual component in DCB. Diferulic acid was summarized as the amounts of 8-O-4′, 8,5′-benz, 5,5′, and 8,5′ dehydrodimers of ferulic acid. Other forms of dehydrodimers of ferulic acid, including 8,8′-diferulic acid, were not identified. nd, not determined; <dl, below detection limit. [b] Estimated loss is calculated values (the values are the relative losses for each component and do therefore not add up to 100%).

Pretreatment. Sixty grams of destarched corn bran was suspended in 1 L of water and pretreated at 190 °C with a 10 min holding time. Heating and cooling periods together added up to 6 min. The pretreatment was conducted in a loop autoclave at Risø DTU as described in refs *28* and *29*. No chemicals or gases were added to the suspension either before, during, or after the treatment. Afterward, the material was separated by filtration into insoluble and soluble fractions that were characterized and enzymatically hydrolyzed individually.

Biomass Composition. Arabinose and xylose were determined after acid hydrolysis with 0.4 M HCl according to the method given in ref *30*.

Cellulose, acetic acid, and lignin were determined after sulfuric acid hydrolysis according to the procedure of NREL (*29*). Sulfuric acid hydrolysis as described by NREL was chosen for acetic acid determination because comparison with alkaline saponification results showed higher release. All samples were analyzed for cellulose and acetic acid content, whereas lignin was determined only in the insoluble samples. Lignin was determined (on nonextracted samples) as Klason Lignin (*29*).

Ferulic acid and four of the dehydrodimers of ferulic acid, namely, 8-O-4′, 8,5′-benz, 5,5′, and 8,5′ dehydrodimers, were quantified by RP-HPLC after alkaline saponification with 2 M NaOH in two successive steps (*31*).

Protein Determination. Protein content was determined according to the method given in ref *32* as total amino acid assessment after 6 M hydrochloric acid hydrolysis followed by separation by ion exchange chromatography.

Enzymatic Hydrolysis. All enzymatic hydrolysis experiments were performed at a 2% DM concentration in 0.1 M succinate buffer, pH 5, and incubated at 50 °C for 24 h with mixing at 1400 rpm. Samples were withdrawn after 0, 2, 4, 6, and 24 h. After incubation, the samples were inactivated immediately at 100 °C for 10 min. Enzymes were loaded according to their enzyme protein (EP) concentration with 0.25 g of EP/kg of DM for each of the hemicellulases (endoxylanase, β-xylosidase, and two α-L-arabinofuranosidases; **Table 1**), 0.5 g of EP/kg of DM for the esterases, and 4 g of EP/kg of DM for the cellulase preparation. All hydrolyses were performed in triplicate.

Analysis (Monosaccharides, Acetic Acid, and Ferulic Acid). Monosaccharides were analyzed using HPAEC-PAD, BioLC Dionex, with a CarboPac PA1 (analytical 4 × 250 mm) column from Dionex according to the method given in ref *30*. Ferulic acid and dehydrodimers of ferulic acid were analyzed using RP-HPLC with DAD detection, Chemstation 1100 series, Hewlett-Packard, and an ODS-L Optimal (250 × 4.6 mm, 5 μm) column from Capital HPLC. The chromatographic profile consisted of buffer A (5% acetonitrile, 1 mM TFA) and acetonitrile starting with 20% acetonitrile. Running gradient was up to 40% acetonitrile for 35 min and further up to 100% for another 3 min, followed by regeneration to 20% acetonitrile for 2 min. Column temperature was maintained at 40 °C. Ferulic acid was detected and quantified at 316 nm using an authentic external standard (Sigma-Aldrich Chemie GmbH, Steinheim Germany) for retention time and spectral recognition; quantification was performed by linear area regression. Dehydrodimers were detected and recognized at 316 nm but quantified at 280 nm according to response factors previously reported (*33*). 5,5′-, 8,5′-, 8-O-4′, and 8,5′-benzofuran diferulic acid were identified. Acetic acid was analyzed by HPLC, Chemstation 1200, Hewlett-Packard, equipped with an Aminex HPX-87H column (300 mm × 7.8 mm) and a refractive index detector. Samples were eluted isocratically with 0.005 M sulfuric acid at 0.6 mL/min at 60 °C for 50 min.

Heat Stability of Acetyl Xylan Esterase. The heat stability of AXE was estimated by incubating the enzyme at 50 °C for up to 24 h immediately followed by activity testing on 500 mg/L pNP-acetate. The assay was conducted as an online spectrophotometrical measurement at 410 nm, 50 °C, and pH 5 in 0.1 M succinate buffer. The enzyme was loaded to a concentration of 10 mg of EP/L, and the assay was performed over a period of 10 min.

Table 3. Ratios of Different Substitution Groups of Corn Bran Arabinoxylan before and after Pretreatment[a]

	DCB	insoluble	soluble
arabinose/xylose	0.71	0.42	0.66
acetic acid/xylose	0.26	0.21	0.42
ferulic acid/arabinose	0.08	0.10	0.09
diferulic acid/arabinose	0.03	0.04	
total substitution on xylan	0.97	0.63	1.08

[a] DCB, destarched corn bran; insoluble, insoluble fraction after pretreatment of DCB; soluble, soluble fraction after pretreatment of DCB. Total substitution on xylan was calculated as the sum of arabinofuranosyl and acetyl substitution. A value of 1.0 in substitution indicates that hypothetically each xylopyranosyl unit carried one substitution unit.

RESULTS

Biomass Composition. Table 2 gives an overview of the monomeric constituents, and **Table 3** gives a proposed substitution pattern of the original destarched corn bran (DCB) and of the soluble and insoluble fractions after pretreatment. The destarched corn bran was mainly composed of arabinoxylan, constituting 56% of the dry matter calculated as the sum of xylose and arabinose with 26.7% arabinose and 37.4% xylose (**Table 2**). The A:X ratio was therefore 0.71. Besides arabinose, acetic acid was also detected, and when calculated on a molar basis, the acetic acid/xylose ratio in the DCB was 0.26, calculated as

$$\left(\frac{38.5\text{ g of acetic acid/kg of DM}}{60\text{ g of acetic acid/mol}}\right)/\left(\frac{374\text{g of xylose/kg of DM}}{150\text{g of xylose/mol}}\right) = 0.26$$

All substitution ratios were calculated on the basis of the hydrated values for all components, because all components were measured as hydrated molecules.

Combined with the arabinose substitution, this gave a total substitution on the xylan backbone of almost 100%, meaning that hypothetically each xylopyranosyl residue held one substitution. This value may be overestimated if arabinofuranosyls as proposed previously (*4*) had been present in short chains instead of as terminal arabinofuranosyl substitutions. Ferulic acid and dehydrodimers of ferulic acid were also found in DCB and constituted ∼4.4% of the biomass (**Table 2**). The hydroxycinnamates were presumably esterified to arabinofuranosyl, resulting in approximately 10% of the arabinofuranosyl units being substituted with either feruloyl or diferuloyl units. All glucose was assumed to originate from cellulose, making cellulose the other

Table 4. Arabinose, Xylose, Acetic Acid, and Ferulic Acid Release after 24 h of Enzymatic Hydrolysis of Pretreated Corn Bran (Insoluble and Soluble Fractions)[a]

	insoluble				soluble			
	arabinose[b]	xylose[b]	acetic acid	ferulic acid[c]	arabinose[b]	xylose[b]	acetic acid	ferulic acid[c]
mini	3.7 (21.2)	1.5 (5.0)			15.2 (48.7)	7.1 (21.0)		
mini + FAE	4.3 (24.4)	1.8 (6.1)		13.7 (64.0)	14.3 (45.7)	9.1 (26.8)		34.4 (91.9)
mini + AXE	4.3 (24.7)	2.9 (9.7)	5.1 (21.4)		15.1 (48.2)	9.5 (28.1)	18.4 (32.8)	
mini + FAE + AXE	4.8 (27.2)	3.1 (10.6)	4.8 (20.0)	14.1 (65.6)	15.4 (49.1)	10.2 (30.0)	16.9 (30.2)	34.2 (91.3)
mini + CTec + FAE + AXE	5.3 (30.1)	4.5 (15.2)	5.5 (23.1)	14.1 (65.5)	14.9 (47.6)	10.5 (35.8)	17.8 (31.8)	34.1 (91.2)

[a] Components are given as percent of each component in the original destarched corn bran. Numbers in parentheses are percent released of each component from the individual fraction. mini, minimal cocktail consisting of endoxylanase, β-xylosidase, and two α-L-arabinofuranosidases; FAE, feruloyl esterase; AXE, acetyl xylan esterase; CTec, Cellic CTec (cellulase preparation). [b] Results given as average of triplicate determinations. Coefficient of variance (CV%) for all samples were in the range of 0.5−9%. [c] Results given as single measurements. Coefficients of variation based on calibration curves were all in the range of 0.2−3%.

major polysaccharide component (21%) in the destarched corn bran.

The presence of lignin (12%) and structural proteins (~9%) completed the impression that corn bran was made up of large complex polymers.

Influence of Pretreatment on DCB. Table 2 also contains the relative composition and mass distribution between each of the two fractions (insoluble and soluble) after pretreatment. The pretreatment resulted in solubilization of arabinoxylan to a certain extent and furthermore altered the composition of the remaining insoluble arabinoxylan. Due to the nature of the pretreatment process, not all material was retrieved from the reactor, causing a certain loss of dry matter (~14%) also estimated in **Table 2**. In particular, arabinose, ferulic acid, and dehydrodimers of ferulic acid were lost. However, up to 16% of the arabinose was liberated as free arabinose, and an additional 31% was solubilized, most likely as oligosaccharides of arabinoxylan because a simultaneous release of xylose occurred after acid hydrolysis. Free xylose was found only in negligible amount. This distribution gave an arabinofuranosyl substitution on the solubilized oligosaccharides of 0.66. The pretreatment also caused changes in the A:X ratio in the insoluble fraction. The A:X changed to 0.42, meaning that the insoluble xylan backbone was now less substituted with arabinose than the original DCB (**Table 3**). The pretreatment presumably did not affect the cellulose and lignin contents as these were found mainly in the insoluble fraction, constituting approximately 73 and 76%, respectively (**Table 2**). The pretreatment had therefore rendered these two components in higher concentrations in the insoluble relative to the original DCB material.

Dehydrodimers of ferulic acid did not seem to endure the pretreatment as almost 75% of the level originally found in the DCB was not confirmed in any of the fractions. Furthermore, as seen from **Table 2** the solubilized material contained almost no dehydrodimers (2% of the original amount), meaning that the insoluble residue was now the only fraction carrying dehydrodimers with up to 0.04 molar substitution on arabinofuranosyls as compared to 0.03 in the original material. Ferulic acid was found both on the solubilized material and in the remaining solids, giving a ferulic acid substitution of approximately 0.09 on the bound arabinose in both the solubilized oligosaccharides and the remaining insoluble fraction. This extent of feruloyl substitution was similar to that of the original destarched corn bran of approximately 0.08 (**Table 3**). The acetyl substitution on xylan was relatively high, with 0.26 in the destarched corn bran, and after pretreatment, most of the acetic acid was found on the solubilized arabinoxylan. In the soluble material, 0.42 of the xylopyranosyl residues were substituted with acetyl, whereas this number was 0.21 for the insoluble remains (**Table 3**).

All in all, this characterized the solubilized xylooligosaccharides as extremely highly substituted with a molar ratio of arabinose and acetic acid to xylose of >1. This extent of substitution was similar to that of the xylan from the original corn bran (0.96, **Table 3**). Several xylopyranosyl moieties must therefore have been doubly substituted, or arabinose may have occurred in short chains on the xylan backbone. The remaining insoluble xylan was merely substituted up to 0.63. Hence, the pretreatment drastically changed the arabinoxylan composition, and further work was evaluated on this basis, treating each fraction as an individual substrate for enzymatic hydrolysis.

Enzymatic Hydrolysis. *Arabinose*. The yields of arabinose tended to increase with addition of the auxiliary enzymes, especially for the insoluble fraction, but the arabinose increases were less pronounced than the xylose increases (**Table 4**). Only minor differences in the arabinose release were seen even when ferulic acid was released to a large extent, and there seemed to be hardly any effect of the simultaneous release of acetic acid either. This trend pointed toward the α-L-arabinofuranosidases not being dependent in their catalytic activity on either deacetylation or deferuloylation or alternatively that the debranching in the case of the insoluble material was not opening the substrate sufficiently to allow access for the relatively large α-L-arabinofuranosidases (65−70 kDa) (*34*). However, the arabinose release in the solubilized material was almost three times as high as that of the insoluble material (**Table 4**), most likely caused by increased enzyme accessibility. The maximal release was in all cases achieved within the first 4−6 h of incubation (data not shown).

Xylose. Enzymatic release of xylose was affected by the addition of acetyl xylan esterase and/or feruloyl esterase. In particular, xylose release in the insoluble fraction (**Figure 2A**; **Table 4**) was promoted by the presence of acetyl xylan esterase and cellulases. When the 24 h data points were compared, it was evident that feruloyl esterase was not responsible for any additional release of xylose compared to the hemicellulases alone, whereas the acetyl xylan esterase caused release of approximately twice the amount of xylose compared to the hemicellulases alone.

In the experiment combining the two types of esterases, the xylose release was not higher than when compared to the acetyl xylan esterase incubated alone with the hemicellulases. Furthermore, the cellulase preparation induced additional release of xylose, and even though the *T. reesei* complex contains β-xylosidase activity (*27*), the increased xylose release was most likely a consequence of simultaneous cellulose degradation.

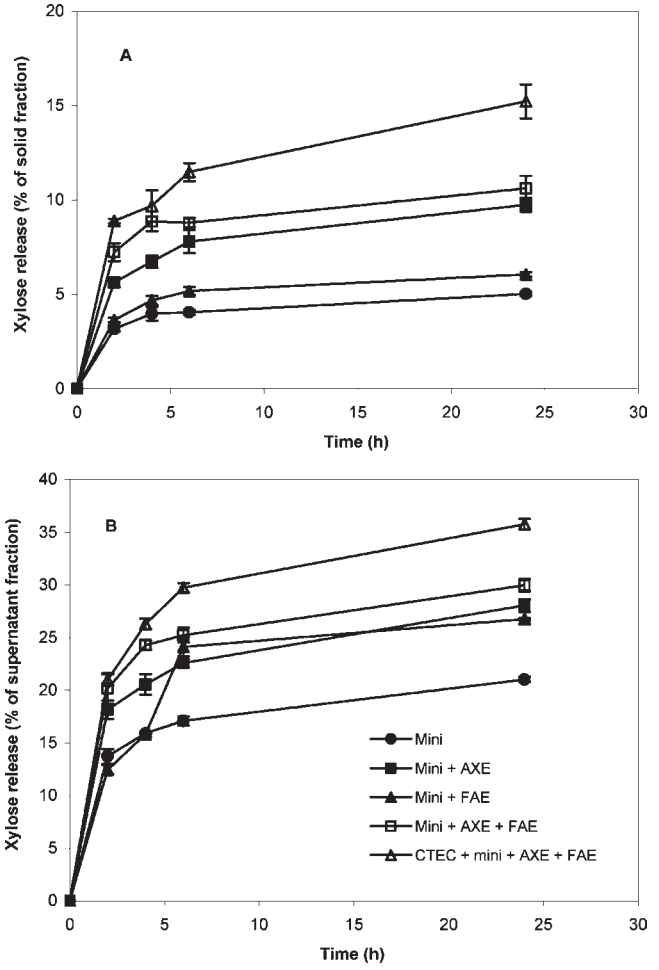

Figure 2. Xylose release from enzymatic hydrolysis of pretreated corn bran in percent of maximum with minimal cocktail (Mini, consisting of endoxylanase, β-xylosidase, and two α-L-arabinofuranosidases), acetyl xylan esterase (AXE), feruloyl esterase (FAE), and cellulase preparation Cellic CTec (CTec): (**A**) insoluble fraction; (**B**) soluble fraction.

The effect of acetyl xylan esterase versus feruloyl esterase was not pronounced to the same degree for the solubilized fraction (**Figure 2B**), indicating that the accessibility for the hemicellulases was not restricted in the same manner as on the insoluble fraction. In addition, the yield of xylose in the solubilized material was higher (up to 36% of the fraction maximum, 10% of the original amount of xylose) than in the insoluble material (approximately 15%, 4% of the original amount of xylose), meaning that the enzymatic accessibility had indeed improved by the solubilization. Despite the low content of cellulose in the soluble fraction (**Table 2**), addition of the cellulase preparation improved the xylose release significantly (**Figure 2B**). Hence, this effect could be due to the particular hydrolysis-boosting protein present in the Cellic CTec preparation or, alternatively, less likely, a result of synergistic interactions between the enzymes in the full blend (Cellic CTec; minimal cocktail, AXE, and FAE). Appropriate controls of esterases incubated alone did not show any release of xylose (data not shown).

Ferulic Acid and Acetic Acid. **Table 4** also shows the release of acetic and ferulic acid after enzymatic hydrolysis. The acetyl xylan esterase was capable of releasing acetic acid equivalent to approximately 20 and 30% of that present in the insoluble and soluble material, respectively. The corresponding releases of ferulic acid by the feruloyl esterase were approximately 65 and 91%. In these experiments no release of diferulic acid was detected, even though the feruloyl esterase from *A. niger* has been known to release especially the 5,5′ and 8-O-4′ dehydrodimers from other substrates such as brewer's spent grain and wheat arabinoxylan (*37*, *38*). However, only high-dosage experiments (10 times) showed detectable release of the 5,5′ dehydrodimer from the soluble and insoluble fractions (data not shown). The acetic and ferulic acid release seemed relatively unaffected by the presence of enzymes other than the esterases. At first, it seemed that the ongoing catalysis by the hemicellulases and cellulases could not provide more substrate for the esterases, but changes in esterase activity might also be the cause of stagnating release, for instance, as a consequence of prolonged incubation at an elevated temperature. Indeed, **Figure 3** shows the result of the heat stability study on AXE and specifies that prolonged incubation at 50 °C significantly reduced the activity of the enzyme. Actually, the enzyme activity was down to

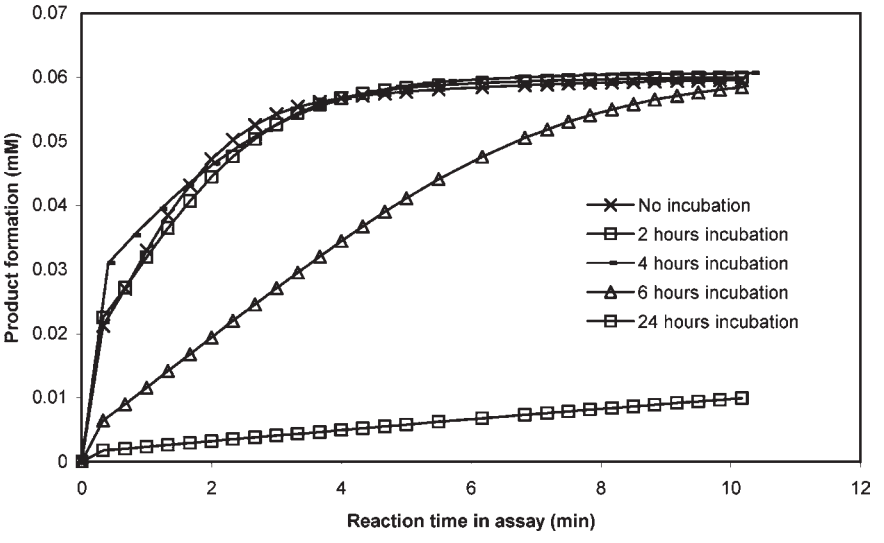

Figure 3. Heat stability of AXE, determined as an activity measurement on pNP acetate after 0−24 h of incubation at 50 °C.

Table 5. Xylose Yield per Released Substituent (Acetic Acid or Ferulic Acid)[a]

	AXE (mol of xylose/mol of acetic acid)	FAE (mol of xylose/mol of ferulic acid)
insoluble	1.06	0.39
soluble	0.51	1.01

[a] Yield expressed as mole equivalents of xylose released per mole equivalent of acetic acid/ferulic acid released when acetyl xylan esterase or feruloyl esterase is added to the minimal cocktail. AXE, acetyl xylan esterase; FAE, feruloyl esterase.

approximately 7% of the original after 24 h of incubation. Appropriate controls incubating acetyl xylan esterase alone or in combination with the hemicellulases did not show any release of ferulic acid. Neither did feruloyl esterase treatment alone or in combination with hemicellulases lead to the release of acetic acid (data not shown).

When the acetic acid and ferulic acid release were compared to the respective xylose release (**Table 5**), it was clear that deacetylation promoted the xylose release to a greater extent than deferuloylation in the insoluble material. Here the reaction ratio was 1:1, leading to the release of 1 mol equiv of xylose for every released mole equivalent of acetic acid (**Table 5**). The corresponding release of ferulic acid was 0.39 mol of xylose released for every mole of ferulic acid (**Table 5**). This fact implied that catalysis by the endoxylanase was entirely dependent on the degree of substitution directly on the xylan backbone rather than on the removal of extended branching. Furthermore, these data support that reactions catalyzed by these types of enzymes (glycosyl hydrolases) could be promoted even on insoluble substrates if the appropriate activities were present and accompanying each other.

The opposite effect was observed for the solubilized oligosaccharides. Here the release of xylose compared to ferulic acid release was 1:1, whereas deacetylation caused only 0.5 mol equiv of xylose/mol equiv of acetic acid. In this case it would have been expected that the arabinose release would have increased equally, but this was not the case. As pointed out previously, arabinose release was affected only marginally by the presence of either of the esterases. As discussed later, this result has several implications, one of them being that the enzymes may be highly dependent on the overall substrate structure rather than just the presence of the bonds to be hydrolyzed.

DISCUSSION

The total release of the different components from both the soluble and the insoluble fractions is summarized in **Table 6** and gives an indication of the remaining material to be hydrolyzed. Addition of esterases to the hemicellulases improved the overall degradation of the corn bran, with addition of AXE showing the highest increase. The cellulase blend further increased the release and therefore the full combination of enzymes released up to 15% of the xylose and 20% of the arabinose present in the original DCB. Assessment of the loss of individual components as a result of the pretreatment was difficult and is based on estimations, but would probably lie in the range of 30−35% for both xylose and arabinose when the total loss of material and the heat lability of these two monosaccharides, in particular, are considered. Taking the loss into consideration 50−55% of the xylose and 45−50% of the arabinose were still not released. A higher dosage of enzymes might further increase the yield as seen in ref *19*, and a test with a 10 times higher dosage as compared to the results reported was performed. The increased dosage did release more arabinose and xylose from the soluble material; however, the effect on the insoluble fraction was negligible (data not shown). In the case of the soluble material, it might be relevant to operate at higher

Table 6. Total Conversion of Pretreated Corn Bran after 24 h of Enzymatic Hydrolysis[a]

	xylose	arabinose	acetic acid	ferulic acid
mini	8.6	19.0		
mini + AXE	12.4	19.4	23.5	
mini + FAE	10.9	18.6		48.1
mini + AXE + FAE	13.3	20.1	21.6	48.2
mini + CTEC + AXE + FAE	15.0	20.2	23.3	48.2

[a] Components given as percent of the original amount in destarched corn bran. mini, minimal cocktail consisting of endoxylanase, β-xylosidase, and two α-L-arabinofuranosidases; FAE, feruloyl esterase; AXE, acetyl xylan esterase; CTec, Cellic CTec (cellulase preparation).

enzyme dosages, but this would have less industrial relevance and might, therefore, not be a feasible option. The pretreatment caused loss of valuable monosaccharides, so avoiding pretreatment would be preferable, but previous studies have shown that enzymatic hydrolysis on untreated corn bran can be exceptionally difficult (*18*), even though relevant activities were applied. The pretreatment used in the experiments presented here was based upon the findings of ref *19*. Here thermal pretreatment promoted the release of xylose at temperatures above 180 °C by significantly solubilizing the biomass and rendering the insoluble residue more accessible to enzymatic attack. Loss of constituents in ref *19* was in the same range as that obtained in the experiments presented here. The relatively large losses of feruloyl (~41%) and diferuloyl (~73%) substituents caused by the pretreatment are of particular relevance to these experiments (**Table 2**). The loss was somewhat disproportional to the loss of other components and could be due to heat lability. The experimental setup and the data obtained did not allow us to provide any firm conclusions about the reason and origin of the ferulic acid losses. Despite the good results obtained so far, a more favorable pretreatment method appears to be desirable to make the biomass less recalcitrant to enzymatic attack without destroying valuable components.

The results showed that pretreating the substrate positively affected the enzyme accessibility on both fractions of the biomass. Of exceptional interest was the finding that acetyl xylan esterase could promote the xylose release from the insoluble corn bran to such a high degree as compared to the promotion induced by feruloyl esterase. As pointed out previously, the effects of deacetylation indicated that the endoxylanase activity toward the insoluble substrate was highly dependent on the exposure of the xylan backbone even when some arabinofuranosyl substitutions remained. The relatively little effect of exposing arabinofuranosyls by deferuloylation could be the result of either low arabinose content in general or the fact that the deferuloylation in the insoluble material did not cause sufficient opening of the substrate for the relatively large arabinofuranosidase molecules (*34*). Usually it is recognized that feruloyl substitutions and cross-linkings hinder enzymatic (*14*) attack, but the experiments presented here and previously (*25*) speak to the fact that acetyl substitution plays an even more important role in the resistance toward enzymatic xylose release from insoluble xylan. Acetyl substitutions have for a long period of time been neglected in the literature, but AXE could be included with success in the battery of enzyme activities necessary to open and degrade corn bran.

The opposite effect of AXE and FAE activity in depolymerizing the solubilized oligosaccharides without the concomitant release of arabinose clearly envisions the diversity of the enzymatic attack performed by the same enzymes. The solubilized oligosaccharides represent a completely different substrate both with respect to substitution pattern, degree of substitution, and most probably also the degree of polymerization (DP) as

compared to the insoluble material. The increased release of xylose occurring with deferuloylation without the simultaneous release of arabinose could indicate that the feruloyl substitutions hindered the endoxylanase and/or β-xylosidase action differently from steric hindrance alone. The endoxylanase used here was from the GH10 family of xylanases known to attack close to substitutions (*39*), and even though the study in question focused on glucuronoxylans, this particular catalytic capability of GH10 xylananses was also shown on heteroxylans and acetylated xylans. However, hydrophobic interactions, such as feruloylation, could possibly influence how close to arabinofuranosyl substitutions the GH10 xylanase would attack the substrate, and deferuloylation would therefore remove this obstacle for xylose release. Combined with the presumed low DP of the substrate, this could perhaps make it possible for the endoxylanase to associate with the substrate closer to arabinofuranosyl substitutions compared to the distance necessary on the insoluble substrate. The lack of arabinose release further brings into question the significance of the present α-L-arabinofuranosidases. However, if arabinofuranosyl substitutions were to some degree made up of short chains rather than entirely of single-moiety substitutions (*4*, *6*), ferulic acid may be positioned along these short chains. The release of ferulic acid from this position would then in turn not render the arabinofuranosyl substitutions available for the α-L-arabinofuranosidases, and therefore simultaneous release of arabinose would not occur. It could also be speculated that ferulic acid was not solely esterified to arabinose but also to xylose in the same manner as acetyl residues. The release of ferulic acid would then open the xylan backbone for endoxylanase attack. Yet no previous suggestions toward this argument have been reported in the literature, and it therefore seems less plausible.

These results also signified that the degree of substitution on the poly-/oligomeric substrates was not necessarily a hindrance for the release of ferulic acid as postulated previously (*21*, *22*). In these experiments it has been evident that the highly substituted material was more accessible to both esterases. However, the accessibility was most likely related to the solubility of the substrate rather than the degree of substitution. Even though the extent of substitution had declined in the insoluble arabinoxylan as a result of pretreatment, it might have only resulted in a limited boost in enzymatic accessibility. Findings have shown that longer stretches of unsubstituted xylan could precipitate as a result of hydrogen bonding (*24*) and thereby leave the substrate enzymatically unavailable. Furthermore, the results here clearly show that the general enzymatic degradation was higher on the soluble substrate and that the initial reaction rate of AXE was also highest when the substrate was soluble (data not shown). The results demonstrate that the tight cooperation of different enzyme activities on polymeric substrates can make even the smallest, seemingly insignificant, activity the link that sustains the continued action of all the other enzymes and that substrate solubility is a vital factor for enzyme accessibility. Future work will have to focus on matching new enzymes to the present recognized battery of arabinoxylan-degrading enzymes and work toward increasing the substrate solubility without the loss of valuable components.

ACKNOWLEDGMENT

We acknowledge Jacob Skibsted Jensen for his participation in acetic acid determinations, and we are grateful to Tomas Fernqvist, Risø DTU, and Ove Skov Nielsen, Novozymes A/S, for assisting in the pretreatment and destarching of corn bran, respectively.

LITERATURE CITED

(1) http://faostat.fao.org/site/339/default.aspx (downloaded 2009.10.28).
(2) http://www.adm.com/company/Documents/ADM_and_Biofuels_Brochure_lores.pdf (downloaded 2009.10.28).
(3) Saulnier, L.; Thibault, J. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **1999**, *79*, 396–402.
(4) Chanliaud, E.; Saulnier, L.; Thibault, J. Alkaline extraction and characterisation of heteroxylans from maize bran. *J. Cereal Sci.* **1995**, *21*, 195–203.
(5) Saulnier, L.; Vigouroux, J.; Thibault, J. Isolation and partial characterization of feruloylated oligosaccharides from maize bran. *Carbohydr. Res.* **1995**, *272*, 241–253.
(6) Saulnier, L.; Marot, C.; Chanliaud, E.; Thibault, J. F. Cell wall polysaccharide interactions in maize bran. *Carbohydr. Polym.* **1995**, *26*, 279–287.
(7) Williamson, G.; Kroon, P. A.; Faulds, C. B. Hairy plant polysaccharides: a close shave with microbial esterases. *Microbiology* **1998**, *144*, 2011–2023.
(8) Smith, M.; Hartley, R. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. *Carbohydr. Res.* **1983**, *118*, 65–80.
(9) Bunzel, M.; Ralph, J.; Marita, J. M.; Hatfield, R. D.; Steinhart, H. Diferulates as structural components in soluble and insoluble cereal dietary fibre. *J. Sci. Food Agric.* **2001**, *81*, 653–660.
(10) Allerdings, E.; Ralph, J.; Schatz, P. F.; Gniechwitz, D.; Steinhart, H.; Bunzel, M. Isolation and structural identification of diarabinosyl 8-O-4-dehydrodiferulate from maize bran insoluble fiber. *Phytochemistry* **2005**, *66*, 113–124.
(11) Bunzel, M.; Ralph, J.; Brüning, P.; Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *J. Agric. Food Chem.* **2006**, *54*, 6409–6418.
(12) Hatfield, R.; Ralph, J.; Grabber, J. Cell wall cross-linking by ferulates and diferulates in grasses. *J. Sci. Food Agric.* **1999**, *79*, 403–407.
(13) Ralph, J.; Bunzel, M.; Marita, J. M.; Hatfield, R. D.; Lu, F.; Kim, H.; Schatz, P. F.; Grabber, J. H.; Steinhart, H. Peroxidase-dependent cross-linking reactions of *p*-hydroxycinnamates in plant cell walls. *Phytochem. Rev.* **2004**, *3*, 79–96.
(14) Grabber, J. H.; Ralph, J.; Hatfield, R. D. Ferulate cross-links limit degradability of lignified primary walls of maize. *J. Agric. Food Chem.* **1998**, *46*, 2606–2614.
(15) Bunzel, M.; Ralph, J.; Lu, F.; Hatfield, R. D.; Steinhart, H. Lignins and ferulate-coniferyl alcohol cross-coupling products in cereal grains. *J. Agric. Food Chem.* **2004**, *52*, 6496–6502.
(16) Lapierre, C.; Pollet, B.; Ralet, M. C.; Sauliner, L. The phenolic fraction of maize bran: evidence for lignin-heteroxylan association. *Phytochemistry* **2001**, *57*, 765–772.
(17) Piber, M.; Koehler, P. Identification of dehydro-ferulic acid-tyrosine in rye and wheat: evidence for a covalent cross-link between arabinoxylans and proteins. *J. Agric. Food Chem.* **2005**, *53*, 5276–5284.
(18) Faulds, C. B.; Kroon, P. A.; Sauliner, L.; Thibault, J. F.; Williamson, G. Release of ferulic acid from maize bran and derived oligosaccharides by *Aspergillus niger* esterases. *Carbohydr. Polym.* **1995**, *27*, 187–190.
(19) Saulnier, L.; Marot, C.; Elgorriaga, M; Bonnin, E.; Thibault, J. F. Thermal and enzymatic treatments for the release of free ferulic acid from maize bran. *Carbohydr. Polym.* **2001**, *45*, 269–275.
(20) Saha, B.; Bothast, R. Pretreatment and enzymatic saccharification of corn fiber. *Appl. Biochem. Biotechnol.* **1999**, *76*, 65–77.
(21) Bartolome, B.; Faulds, C.; Williamson, G. Enzymic release of ferulic acid from barley spent grain. *J. Cereal Sci.* **1997**, *25*, 285–288.
(22) Faulds, C.; Bartolomé, B.; Williamson, G. Novel biotransformations of agro-industrial cereal waste by ferulic acid esterases. *Ind. Crop Prod.* **1997**, *6*, 367–374.
(23) Grohmann, K.; Mitchell, D. J.; Himmel, M. E.; Dale, B. E.; Schroeder, H. A. The role of ester groups in resistance of plant cell wall polysaccharides to enzymatic hydrolysis. *Appl. Biochem. Biotechnol.* **1989**, *20*, 45–61.

(24) Poutanen, K.; Sundberg, M.; Korte, H.; Puls, J. Deacetylation of xylans by acetyl esterases of *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* **1990**, *33*, 506–510.
(25) Biely, P.; Mackenzie, C. R.; Puls, J.; Schneider, H. Cooperativity of esterases and xylanases in the enzymatic degradation of acetyl xylan. *Nat. Biotechnol.* **1986**, *4*, 731–733.
(26) Rosgaard, L.; Pedersen, S.; Langston, J.; Akerhielm, D.; Cherry, J. R.; Meyer, A. S. Evaluation of minimal *Trichoderma reesei* cellulase mixtures on differently pretreated barley straw substrates. *Biotechnol. Prog.* **2007**, *23*, 1270–1276.
(27) Sørensen, H.; Pedersen, S.; Viksø-Nielsen, A.; Meyer, A. S. Efficiencies of designed enzyme combinations in releasing xylose and arabinose from wheat arabinoxylan in an industrial ethanol fermentation residue. *Enzyme Microbial. Technol.* **2005**, *36*, 773–784.
(28) Bjerre, A. B.; Olesen, A. B.; Fernqvist, T.; Plöger, A.; Schmidt, A. S. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnol. Bioeng.* **1996**, *49*, 568–577.
(29) Pedersen, M.; Meyer, A. S. Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw. *Biotechnol. Prog.* **2009**, *25*, 399–408.
(30) Sørensen, H. R.; Meyer, A. S.; Pedersen, S. Enzymatic hydrolysis of water-soluble wheat arabinoxylan. 1. Synergy between α-L-arabinofuranosidases, endo-1,4-β-xylanases, and β-xylosidase activities. *Biotechnol. Bioeng.* **2003**, *81*, 726–731.
(31) Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, A. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. *J. Agric. Food Chem.* **2000**, *48*, 2837–2842.
(32) Barkholt, V.; Jensen, A. L. Amino acid analysis: determination of cysteine plus half-cysteine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Anal. Biochem.* **1989**, *177*, 318–322.
(33) Waldron, K. W.; Parr, A. J.; Ng, A.; Ralph, J. Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem. Anal.* **1996**, *7*, 305–312.
(34) Sørensen, H. R.; Pedersen, S.; Jørgensen, C. T.; Meyer, A. S. Enzymatic hydrolysis of wheat arabinoxylan by a recombinant "minimal" enzyme cocktail containing β-xylosidase and novel endo-1,4-β-xylanase and α-L-arabinofuranosidase activities. *Biotechnol. Prog.* **2007**, *23*, 100–107.
(35) Faulds, C. B.; Williamson, G. Purification and characterization of a ferulic acid esterase (FAE-III) from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcrystalline cellulose. *Microbiology* (*Reading, U.K.*) **1994**, *140*, 779–787.
(36) Faulds, C. B.; Williamson, G. Release of ferulic acid from wheat bran by a ferulic acid esterase (FAE-III) from *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 1082–1087.
(37) Faulds, C. B.; Sancho, A. I.; Bartolomé, B. Mono- and dimeric ferulic acid release from brewer's spent grain by fungal feruloyl esterases. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 489–493.
(38) Faulds, C. B.; Zanichelli, D.; Crepin, V. F.; Connerton, I. F.; Juge, N.; Bhat, M. K.; Waldron, K. W. Specificity of feruloyl esterases for water-extractable and un-extractable feruloylated polysaccharides: influence of xylanase. *J. Cereal Sci.* **2003**, *38*, 281–288.
(39) Biely, P.; Vrsanská, M.; Tenkanen, M.; Kluepfel, D. Endo-β-1,4-xylanase families: differences in catalytic properties. *J. Biotechnol.* **1997**, *57*, 151–166.

Received for review November 15, 2009. Revised manuscript received April 6, 2010. Accepted April 12, 2010. This study was partially financed by Novozymes A/S and FOOD Denmark Graduate School.